Mild prenatal hypoxia-ischemia leads to social deficits and central and peripheral inflammation in exposed offspring.

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Running Title: Preterm hypoxia-ischemia and social deficits.

Abstract

Hypoxic-ischemic encephalopathy (HIE) resulting from intrauterine or perinatal hypoxic-ischemia (HI) is a leading cause of long-term neonatal neurodisability. While most studies of long-term outcome have focused on moderate and severe HIE in term infants, recent work has shown that those with mild HIE may have subtle neurological impairments. However, the impact of mild HI on pre-term infants is much less clear given that pre-term birth is itself a risk factor for neurodisability. Here we show that mild HI insult alters behaviour, inflammation and the corticosterone stress response in a rat model of pre-term HIE. Mild HI exposure led to social deficits in exposed offspring at postnatal day 30, without impairments in the novel object recognition test nor in the open field test. This was also accompanied by elevations in circulating adrenocorticotropic hormone and corticosterone indicating an exaggerated stress response. There were also elevations in *il-1β* and *il-6* but not *tnf-α* mRNA and protein in the brain and blood samples. In summary we find that a mild HI exposure leads to social deficits, central and peripheral inflammation, and an abnormal corticosterone response which are three core features of autism spectrum disorder. This shows that mild HI exposure may be a risk factor for an abnormal neurodevelopmental outcome in pre-term offspring.

Keywords: Hypoxic-ischemic brain injury; HIE; Perinatal asphyxia; Autism; ASD; Inflammation; Interleukin-6; Corticosterone; Stress.

1. Introduction

Hypoxic-ischemic encephalopathy (HIE) is a leading cause of death and long-term disability in neonate, hypoxic-ischemic encephalopathy is a consequence of a hypoxic insult arising from intrauterine or perinatal asphyxia (Locatelli et al., 2008, McIntyre et al., 2013, Harteman et al., 2013). This may be due to prolonged partial asphyxia secondary to reduced placenta blood flow, prolonged or obstructed labour, or an acute sentinel event such as uterine rupture, placental abruption or acute cord occlusion (Volpe, 2012). This leads to reduced cerebral perfusion which results in an inadequate supply of oxygen and glucose to the developing brain (Armstrong et al., 2012, Barberi et al., 1999). Reduced cerebral perfusion combined with hypoxia is known as hypoxia-ischemia (HI) (Fatemi et al., 2009). If a HI event damages the developing brain, an evolving encephalopathy is seen that is clinically graded as mild, moderate or severe (Fatemi et al., 2009, Shah and Perlman, 2009, Volpe, 2012).

The patterns of brain injury that result from HIE, and the long-term neurological outcomes depend on the grade of HIE and on the gestational age at the time the insult occurs (Logitharajah et al., 2009, Cabaj et al., 2012). The majority of studies on long-term neurodevelopmental outcome have focused on term infants with moderate and severe HIE, and have reported increased rates of a range of motor and non-motor neurological disability, (Mwaniki et al., 2012) including cognitive impairments (Lindstrom, Hallberg et al. 2008) and epilepsy (Kharoshankaya et al., 2016). Term infants with mild HIE have been considered to have a normal outcome, however, recent work has shown that these infants have cognitive impairments at five years (Murray et al., 2016). There is also some evidence that HI conditions increase the risk of autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD) in exposed children (van Handel et al., 2007, Getahun et al., 2013, Getahun et al., 2013). Multiple studies have reported that prenatal and perinatal complications that can cause HI are associated with an increased risk of ASD. In particular, recent work in human populations has shown that neonatal respiratory distress and other markers of hypoxia were associated with increased risk of ASD in males in a twin study (Froehlich-Santino et al., 2014). Moreover, a recent meta-analyses also demonstrated an increased risk of ASD in children with neonatal hypoxia (Modabbernia et al., 2016).

In contrast to term infants, studying long-term neurodevelopment outcome in preterm infants (those born <37 weeks) with HIE is more difficult given that preterm birth is itself a

risk factor for a poor neurodevelopment outcome and often occurs with co-existent pathologies. The result of this is that the contribution of mild HI exposure to long-term neurodevelopmental outcomes in preterm infants is unclear (Gopagondanahalli et al., 2016). This is particularly difficult in very low gestational age (VLGA) infants (born <32 weeks') which occurs in 1% of singleton and 9% of twin pregnancies (Schaaf et al., 2011). Modelling HI exposure in VLGA infants has been carried out in rodents by examining the impact of HI exposure on the rat brain just before birth. This is because key neurodevelopmental processes that occur in humans from 23-32 weeks of gestation occur in the rat brain around birth (Semple et al., 2013). Previous work in these rat models of preterm HIE have examined the functional impact primarily on three core domains; motor deficits, social deficits and anxiety (Vazquez-Borsetti et al., 2016) and cognition and learning (Saraceno et al., 2016, Barkhuizen et al., 2017). While the functional impact of preterm exposure to moderate and severe HI has been the focus of intensive investigation (Barkhuizen et al., 2017), given the emerging clinical picture showing that term infants with mild HIE have subtle, long-term impairments without overt neurological injury (Murray et al., 2016), there is a need to understand the contribution of mild-HI exposure to the long-term neurodevelopmental outcomes in preterm offspring.

2. Materials and Methods

2.1 Study 1: Generation of a model of a mild pre-term HI insult.

2.1.1 Animals and study design.

All work was carried out under licence with ethical approval from the institutional ethics committee. Animals were maintained in a controlled environment on a 12 h light/dark cycle (lights on at 7:30 am) with *ad libitum* access to food and water. All experiments were performed in a blinded manner. Pregnant Sprague-Dawley (SD) rats were randomised into one of the following groups: (1) caesarean delivery, with no hypoxia exposure (sham, GD 22); (2) caesarean delivery following a 3, 5 or 7 min HI insult where indicated (Fig. 1). For the induction of HI, pregnant dams were induced and maintained under isoflurane (2%) anaesthesia and 21% oxygen (O_2). Following laparotomy, the infra-renal abdominal aorta and uterine arteries were ligated. The inhaled O_2 concentration was reduced to 10% for 3, 5 or 7 min to induce hypoxia. Durations longer than 7 min lead to a significant increase in offspring mortality and so were discontinued. After the HI exposure the O_2 concentration was restored from 10% to 21% to restore blood flow and O_2 saturation to induce a reperfusion injury. The

pups were then immediately delivered by caesarean section (C-section) on GD 22, and manually stimulated to initiate breathing in an incubator at 37ºC for 1h in room air. Two pups (males only) from each dam were cross fostered to a foster mother post-delivery. These foster mothers were first time mothers with foster pups only, litter sizes (n = 9-12). Maternal bonding behaviour was observed initially for rejection which was defined as any aggressive behaviour towards a pup, avoidance to nest or rejection to allow to feed. Pup weight was used as a measure to determine if feeding rejection occurred.

2.1.2 Western blotting

To assess caspase-3 activation, brains were collected on dry ice at postnatal day five (P5). Postnatal day five was chosen to assess death to encompass the combination of acute injury (24-48 hours) and delayed injury response (3-5 days). Proteins homogenates of the whole brain from one pup, from three individual litters (n=3 per group) were prepared using RIPA buffer containing a protease inhibitor cocktail (Santa Cruz Biotech., USA). Proteins were resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane, that was blocked in 5% BSA in PBST (10mM PBS + 0.1% Tween20), and incubated with primary antibodies against cleaved caspase-3 (1:500); pro-caspase-3 (1:1000); or β-actin (1:1000) (all Sigma) in 1% BSA in PBS-T for 16 h at 4°C. Following washes, membranes were incubated with HRP-conjugated secondary antibodies (1:2000, Sigma) and were detected with the ECL-Plus system (Amersham). Images were analyzed by densitometry using Image J (NIH Image J 1.47v). Protein expression was normalized to that of β-actin.

2.1.3 TUNEL staining

To assess cell death in the tertiary phase of injury, we carried out terminal deoxynucleotidyl transferase dUPT nick-end labelling (TUNEL) staining in an additional cohort of offspring at P20. We used 2 male pups per litter from 5 litters (n=10 per group). To do this the offspring were anaesthetised at P20, and fixed by transcardial perfusion of 4% paraformaldehyde. The offspring were then culled by rapid decapitation, their brains were extracted and then post-fixed for 24 h at 4°C in 4% paraformaldehyde. Following cryoprotection in a 30% sucrose in 10mM PBS for 24 h, samples were snap frozen and sectioned at 20µm intervals. TUNEL staining was performed according to the manufacturer's instructions (ApopTag; Millipore, USA). Five fields were randomly chosen in the CA1, CA2 and CA3 regions of the hippocampus and the numbers of TUNEL-positive were counted. Data are presented as the mean number of TUNEL-positive cells per field of view.

2.1.4 28-point neuroscore testing

Sensorimotor function was assessed using a modified version of the 28-point neuroscore (Encarnacion et al. 2011) in two additional cohorts; one of which was assessed at P20-P25, and one at P30-35. We used 2 male pups per litter from 5 litters (n=10 per group). The neuroscore test consisted of 11 tests with a maximum score of 28 (Table 1). Scoring was determined on a scale from 0 (impairment) to 28 (no impairment). Each animal was observed during each task at either P20-25 or P30-35. The 28-point neuroscore was completed for 5 days, and the mean cumulative score calculated per time period was calculated.

2.2 Study 2: Behavioural and molecular outcomes in the mild model of pre-term HI.

2.2.1 Animals and study design

The rest of the study consisted of new cohorts of offspring that were randomised into three experimental groups: (1) offspring born by a normal vaginal delivery on GD22/23 (vaginal); (2) those born by C-Section without hypoxia on GD22 (C-section); and (3) those born by C-section following exposure to 3 min HI *in utero* (C-section + 3 min HI). (Fig. 1) Two pups (males only) from each dam were cross fostered to a foster mother post-delivery and monitored as outlined in Section 2.1.1. Pup weights were recorded at P1, P7, P14, P21 and P30 prior to behavioural analysis and tissue sampling at P30. For all the behavioural testing we used 1-2 male pups per litter from 5-6 litters (n=10 per group).

2.2.2 The social avoidance test

The social-avoidance test (SAA) was performed in a white open field (40 x 40 cm) containing a wire mesh cage (10 x 6 cm) located at one end of the arena in a dark room. Each rat received two consecutive 2.5 min exposure sessions to the arena. The first session consisted of "no target" and the second session consisted of "target". The social target was an unfamiliar male Sprague Dawley rat. Between sessions, each rat was returned to the home cage for one min. The latency (s) to approach the target, the number of entries to the "interaction zone" (a 6 cm corridor surrounding the cage), and time (s) in the interaction zone were observed.

2.2.3 Open field and novel object recognition testing

Open field and novel objection recognition (NOR) was performed as previously described (Straley et al., 2017). Briefly, locomotor activity was assessed using the open field test,

briefly, this consisted of a square arena, divided into 25 equal-sized squares and distance, duration of movement, number of squares entered was counted for five min. Exploratory behaviour and the ability to recognize a novel object was assessed using the NOR test. Briefly, a rat was familiarised with an arena (no objects) for five minutes per day for three consecutive days. On the fourth day, the rats were placed in an arena which contained two similar objects. The first 10 min of exploration was recorded. On the fifth day, the recognition test was performed, one familiar and one novel object, using same location as objects previously. Exploratory behaviour was recorded for 3 min. A positive approach was scored when the rat was within 2 cm of the object and illustrating direct interest.

2.2.4 Brain and blood sample collection and ELISA

On P30, blood samples were collected by cardiac puncture within 1 h of completing the SAA test. 100 µl of blood was collected and centrifuged at 1000g x 10 min. The serum was removed and undiluted samples were stored at -80°C prior to analysis. At P30, animals were also perfused with saline to remove the blood, and brain regions (whole prefrontal cortex, hippocampus and hypothalamus) were microdissected and stored at -80°C. ELISA analysis was performed using commercially-available ELISA kits for IL-6, TNF-α, IL-1β, corticosterone, and adrenocorticotropic hormone (ACTH) according to manufacturer instructions (Abcam). For all ELISA testing, we used 1-2 pups per litter, 3-5 litters (n=6-8).

2.2.3 Quantitative PCR

RNA was extracted using an RNeasy mini kit (Qiagen). For reverse transcription, 200ng mRNA was reverse transcribed in a reaction mixture consisting of 2µl of oligonucleotide (dT, 500 µg/ml), 2 µl DTT (0.1 M), dNTP mix, 2 µl 10x RT random primers, 1 µl reverse transcriptase and 4 µl nuclease-free H_2O (Applied Biosystems) at 25°C for 10 min; 37°C for 120 min; 85°C for 5 min; and 4°C for 10 min. All samples were then run in triplicate using the StepOne Real-Time PCRY System (Applied Biosystems). Each reaction contained a mixture of 1 µl cDNA, 10 µl master mix, 2 µl primers (TaqMan), and 8 µl RNase-free H_2O in a 96 well PCR reaction plate (Applied Biosystems). The primer and probe sequences for amplification of each cDNA were; *il6* F: 5'ACCTGCCTGCTGAGAATCACT'3; R: 5'TTGGCTCTGTAACAGGGGATAT'3, *tnf-α* F: 5'AGTCCCCAAACAACCTCCAT'3 R: 5'TTGACCGCTGAAGAGAACCT'3, *il-1β* F: 5' TGAAGCAGCTATGGCAACTG'3 R:5' CTGCCTTCCTGAAGCTCTTG'3 and *gapdh* F: 5'TGGCACAGTCAAGGCTGAGA'3

R: 5'CTTCTGAGTGGCAGTGATGG'3. The cycling parameters for *gapdh* were 10 min at 95°C followed by 40 cycles of: 95°C for 30 s; 55°C for 1 min; 72°C for 1 min. The cycling parameters for *il*-6, *tnf-*α and *il-1*β were 10 min at 95°C followed by 35 cycles of: 95°C for 5 min; 94°C for 15 s; 59°C for 30 s. Data analysis was carried out using the 2-δCT method. For all quantitative PCR we used 1-2 pups per litter, 3-5 litters (n=6-8).

2.3 Statistical Analysis

All data are presented as mean ± SEM and deemed significant when $p<0.05$. Unpaired Student's t-test or one-way ANOVA with a post hoc Tukey's test was performed where appropriate to determine significant differences between groups.

3. Results

3.1 Study 1: Longer duration of HI is increase cell death in the postnatal period.

In order to establish a mild model of preterm HI, we first sought to determine the effect of increasing duration of HI exposure on the activation of apoptotic pathways and cell death in the brains of offspring. Western blotting showed that there was an overall effect of duration of HI exposure on ratio of cleaved Caspase 3 to pro-Caspase 3 in the hippocampi of exposed offspring at P5 ($F_{(3,36)} = 81.31$, $p<0.0001$) with *post-hoc* testing showing significance between the sham and the 3 min ($p<0.01$), 5 min ($p<0.001$) and 7 min groups ($p<0.0001$) (Fig. 2a, b). To determine if this led to increased cell death, we examined the numbers of $TUNEL^+$ cells in the hippocampus of affected offspring at P20. There was a significant effect of HI duration on number of $TUNEL^+$ cells ($F_{(3, 36)} = 203.4$, $p<0.0001$) with a significant increase observed only in the 5 min and 7 min groups ($p<0.001$ in both cases), but not in the 3 min group (Fig. 2c, d). There was a 100% survival rate in the 3 min HI and 5 min HI groups, whereas this dropped to 92% for 7 min HI group (data not shown). These data show that exposure to longer periods of HI results in increased apoptosis in the brains of exposed offspring in the postnatal period.

3.2 Study 1: The duration of HI is the critical determinant of major neurological impairment.

To determine the functional significance of these changes, we next used a modified 28-point neuroscore (Encarnacion, Horie et al. 2011) to assess post-ischemic motor and behavioural deficits. A one-way ANOVA showed an overall effect of HI duration on the neuroscore at P20-25 in one cohort ($F_{(3,36)} = 23.19$, $p<0.0001$) and at P30-35 in an additional cohort ($F_{(3, 36)}$

= 11.56, p<0.0001), with significant deficits evident in the 7 min group compared to sham at P20-P25 (p<0.0001) (Fig. 2e) and P30-P35 (p<0.001) (Fig. 2f). To exclude a confounding effect of reduced body weight which may impact these findings, we also examined weight gain in the postnatal period, but found that this was unaffected by HI exposure (Supplementary Fig. 1). These data show that the duration of HI exposure is the critical determinant of major neurological impairments in exposed offspring. As these data revealed that there was no significant increase in cell death nor evidence of any gross neurological impairment following exposure to 3 min HI, and that these offspring had a normal growth trajectory in the postnatal period (Fig. 1), the 3 min HI exposure was defined as a mild HI exposure. This 3 min HI exposure was then selected to study the impact of mild HI exposure on cognitive and social behaviour, the stress response, and pro-inflammatory cytokine expression in these offspring.

3.2 Study 2: Mild perinatal HI exposure induces social deficits in exposed offspring.

The rest of the study of new cohorts of animals randomised into three experimental groups: (1) animals born by a normal vaginal delivery on GD22/23 (vaginal); (2) those born by C-Section without hypoxia on GD22 (C-section), and (3) those born by C-section section following exposure to 3 min HI *in utero* (C-section + 3 min HI). We first assessed behaviour in the SAA test at P30 (Fig. 1b). The C-section + 3 min HI group spent significantly less time in the interaction zone if a social target was present ($F_{(2, 27)}$ = 17.25, p<0.0001) when compared to either of the other two groups, whereas they showed no preference for any compartment in the absence of a social target (Fig. 3a). The C-section + 3 min HI group also had significantly lower number of entries ($F_{(2, 27)}$ = 14.30, p<0.0001) into the interaction zone only in the presence of a social target when compared to offspring born vaginal or by C-section (Fig. 3b). This was also reflected in the increased latency to enter the interaction zone in the C-section + 3-min HI group compared to offspring born vaginal or by C-section ($F_{(2, 27)}$ = 13.16, p<0.0001) only when a social target was present (Fig. 3c). To avoid a potential confounding effect of multiple behavioural tests, we also assessed the performance in the novel object recognition test ($F_{(2, 33)}$ = 2.79, p = 0.08) and total distance moved in the open field test ($F_{(3, 11)}$ = 1.49, p = 0.27) in a separate group of animals, and found no significant differences in these parameters. These data show that brief exposure to a mild perinatal HI can alter social behaviour in exposed offspring.

3.3 Study 2: Mild perinatal HI exposure alters the stress response in exposed offspring.

Given the social deficits, we next determined whether this was paralleled by molecular changes that have been described in ASD. Firstly, we focused on the cortisol stress response as an enhanced cortisol social stress response has been reported in children with ASD (Spratt et al. 2012). To determine if exposure to mild HI altered the corticosterone stress response, we performed ELISA analyses for ACTH and corticosterone on blood and hypothalamic samples taken immediately after the SAA test. A one-way ANOVA revealed an overall effect of the mild HI exposure on circulating levels of ACTH ($F_{(2, 21)} = 9.21$, $p<0.01$) and circulating levels of corticosterone ($F_{(2, 21)} = 6.77$, $p<0.01$) (Fig. 4a). *Post-hoc* testing revealed a significant increase in the C-section + 3 min HI group compared to the vaginal ($p<0.01$) and C-section groups ($p<0.05$). When we examined the hypothalamus, we found no significant effect of the mild HI exposure on ACTH levels, however there was a significant effect of the mild HI exposure on corticosterone levels ($F_{(2, 21)} = 14.49$, $p = 0.0001$). *Post-hoc* testing revealed that this was elevated in the C-section + 3 min HI group compared to the vaginal ($p<0.001$) and the C-section ($p<0.01$) groups (Fig. 4b). These data show that mild a HI exposure alters the corticosterone stress response in HI-exposed preterm offspring.

3.4 Study 2: Mild perinatal HI leads to central inflammation.

We next determined if there were central changes in cytokine expression similar to those in ASD. To this end we examined the expression of IL-6 and IL-1β which have been shown in a recent systematic review and meta-analysis to be elevated in ASD, and TNF-α which was not (Masi et al., 2015). To do this we analysed the mRNA and protein level of these cytokines in the whole prefrontal cortex (PFC), the hippocampus and in blood samples taken at P30. There was a significant effect of HI exposure on *il-6* mRNA ($F_{(2, 15)} = 6.4$, $p<0.01$) and IL-6 protein ($F_{(2, 17)} = 7.68$, $p<0.01$) levels in the PFC . *Post-hoc* testing revealed a significant increase in the C-section + 3 min HI group compared to the vaginal and the C-section control groups (Fig. 5a). We also found a significant increase in *il-1β* mRNA ($F_{(2, 15)} = 5.03$, $p<0.05$) and IL-1β protein levels ($F_{(2, 16)} = 7.17$, $p<0.01$) in the PFC (Fig. 5b) in the C-section + 3 min HI group compared to the vaginal and the C-section control groups. In contrast, we found no significant differences in *tnf-α* mRNA or TNF-α protein expression between any of the groups (Fig. 5c). We next determined if these changes in cytokine mRNA and protein levels were mirrored in the hippocampus. Similar to the PFC, we sound a significant increase in *il-6* mRNA ($F_{(2, 15)} = 6.244$, $p = 0.01$) and IL-6 protein ($F_{(2, 18)} = 10.26$, $p = 0.001$) (Fig. 5d), and

il-1β mRNA ($F_{(2, 15)} = 6.37$, $p = 0.01$) and IL-1β protein ($F_{(2, 16)} = 5.781$, $p = 0.01$) (Fig. 5e) but not *tnf-α* mRNA or protein (Fig. 5f) in the C-section + 3 min HI group compared to the control groups.

3.4 Study 2: Mild perinatal HI leads to a circulating inflammatory phenotype.

To determine if central inflammatory profile was also reflected in the periphery, we also examined the mRNA and protein levels of these cytokines in the blood of exposed offspring. The elevated IL-6 and IL-1β central cytokine profile was mirrored by similar increases in the periphery. Specifically, there was a significant increase in *il-6* mRNA ($F_{(2, 17)} = 4.08$, $p<0.05$) and IL-6 protein ($F_{(2, 15)} = 5.97$, $p<0.05$) which was significantly elevated in the C-section + 3 min HI group compared to the vaginal and the C-section control groups (Fig. 6a). Similar changes were also found in relation to *il-1β* mRNA ($F_{(2, 21)} = 4.48$, $p<0.05$) and *il-1β* protein ($F_{(2, 15)} = 5.77$, $p<0.05$) (Fig. 6b), but not *tnf-α* mRNA or protein levels (Fig. 6c). These data show that a mild HI exposure results in an on-going central and peripheral immune dysregulation in the PFC and hippocampus of exposed offspring that changes the expression of cytokines known to be altered in ASD.

4.0 Discussion

In this study, we used a perinatal rat model of HI exposure which is a development stage where the rodent brain is most similar to that of a preterm human infant (Semple et al., 2013). Behavioural and molecular changes were assessed between P25 and P30. At this stage the rat brain corresponds to 4 to 11 years of brain development in humans (Semple et al., 2013). Therefore this study was designed to examine the effects of mild preterm HI brain injury on outcomes in early-life in rats at a development stage in which neurodevelopmental disorders are well established in humans (Borre et al., 2014). This is important, as this group of infants is often understudied and there are currently no therapeutic options available. Better understanding of the long-term behaviours and the molecular correlates of the behavioural changes that result from mild HI injury will help identify new therapeutic targets and help inform strategies for the surveillance and management of the outcome in affected children.

We first found that the duration of HI exposure was the critical determinant of gross motor and behavioural impairments as measured using the neuroscore test, where exposure to 7 min HI led to significant impairments. In contrast, exposure to 3 min or 5 min HI led to no significant motor or behavioural impairments in the neuroscore test. These data agree with

preclinical (Van de Berg et al., 2002, Kiss et al., 2009) and clinical data showing that long-term motor and reflex abnormalities are common after moderate and severe HIE (van Handel et al., 2007, Logitharajah et al., 2009, Adhikari and Rao, 2017). The behavioural changes were also supported by the measurements of caspase-3 cleavage and measurements of the numbers of apoptotic cells in the hippocampus at P5 in which we found that 5 or 7 min exposure to HI, but not 3 min led to a significant increase in both of these. In agreement with this work in the perinatal submersion model of HI injury has shown that perinatal asphyxia for 20 minutes (a severe insult in that model) led to a significant increase in the numbers of $TUNEL^+$ cells in the striatum at P8 (Van de Berg et al., 2002). Here we also report social deficits, without an effect on motor or memory function at P30 in offspring exposed to 3 min HI.

Recent evidence in a rat model of HI injury demonstrates sex-specific mitochondrial and oxidative alterations (Demarest et al., 2016), short term cell genesis increase in females (Waddell et al., 2016), and males are more vulnerable to motor and sensory deficits in the long term (Huang et al., 2016). Likewise, treatments for HI injury appears to be sex-dependent, females more than males have an improved response to dual therapy (hypothermia and allopurinol) (Rodriguez-Fanjul et al., 2017). Moreover, Mirza et al, demonstrated that females and males may have similar acute phase injury but that male animals have an up-regulation of the innate immune response providing supporting evidence of sexual dimorphism (Mirza et al., 2015). Similarly, ASD has a male preponderance (Palmer et al., 2017). Given, the notable different sex outcomes post HI injury, and male tendency for social deficits, and the need to exclude pubertal hormonal bias during adolescent social testing, we studied only males in the current study. However, in future work it will be important to clarify if the social deficits, and the biochemical changes are sex specific.

Previous work has shown that a severe HI insult (20 minutes) in the perinatal period in rats results in social deficits and a significant loss of neurons in the offspring at ~P30 (Vazquez-Borsetti et al., 2016). Moreover, P1 rat pups exposed to a single 30 min period of 100% N_2 also displayed social deficits at P15 (Laviola et al., 2004). A mild increase in caspase cell death at P5 without major $TUNEL^+$ cells at P20 in the brief 3-min HI group induced an acute insult sufficient to alter an inflammatory response without initiating a programmed cell death response. This may support a two-hit hypothesis, early HI exposure and protracted exaggerated stress/inflammatory response. Our data show that even in the

absence of brain injury and major neurodevelopment impairments (28-point neuroscore test), mild perinatal HI can lead to social deficits in exposed offspring.

One of the most striking findings from this work is the evidence for elevations in central and peripheral IL-1β and IL-6 (but not TNF-α) levels at P30. While the impact of HI on cytokine expression in the period immediately after the insult has been extensively studied in pre-clinical models and humans, to our knowledge this is the first demonstration that perinatal HI exposure results in a sustained pro-inflammatory state in exposed offspring. Given the social deficits and the increases in IL-1β and IL-6, it is interesting to note that a recent systematic review of cytokine aberrations in ASD reported significant increases in circulating levels of IL-1β and IL-6 (Masi et al., 2015). Moreover the finding that an acute sentinel event can lead to long term cytokine alterations is supported by recent work in a non-human primate model which has shown exposure to a brief period (3 days) of maternal immune activation (MA) resulted in elevated production of innate inflammatory cytokines including both IL-1β and IL-6 in affected offspring one year later (Rose et al., 2016). Increased levels of IL-6 and TNF-α have been reported to correlate with socialization, intelligence and irritability in some clinical studies of ASD (Ferguson et al., 2016). This is in contrast to our study, in which TNF-α is not significant, however most studies suggest larger numbers of well-characterised clinical populations (that accommodate for acute vs chronic stress responses) are required to elucidate the contribution of TNF-α in the pathophysiology of ASD (Guloksuz et al., 2017, Ghaffari et al., 2016). Moreover, there is a large variation in the age (mean age ranges from 2 to 21) of participants in studies investigating cytokine levels in ASD (Tostes et al., 2012, Ricci et al., 2013, Croonenberghs et al., 2002). Increasing evidence suggests that elevated IL-6 may be an important driver of altered behaviour in response to *in utero* adversity (Meyer et al., 2006, Smith et al., 2007, Wu et al., 2016), with recent work showing that blocking IL-6 led to increased sociability in the BTBR mouse model of ASD (Wei et al., 2016). This is interesting considering that there are a number of drugs under investigation as anti-IL-6 therapeutics for various conditions including brain disorders (Fonseka et al., 2015). Whether blocking IL-6 signalling may be a therapeutic approach to improve neurodevelopmental outcome in infants with mild-HIE is an important question for future research.

In addition, it is known that vertical transmission of microbiota is influenced by mode of delivery, and the long term effects are still under investigation (Dominguez-Bello et al., 2010). The microbiome composition is also altered between preterm and full-term infants,

and additionally impacted by mode of delivery such as caesarean section (Forsgren et al., 2017). Moreover, significant evidence is emerging of the cross-talk between mode of delivery, microbiota and neurodevelopmental disorders including ASD (Curran et al. 2016). A recent meta-analysis demonstrates mode of delivery influences healthy gut microbiota and subsequent development and maturation of the immune system (Rutayisire et al., 2016). In the present study, we incorporated a sham group (caesarean only with no HI) to control for mode of delivery as a factor, albeit we did not deliver the animals in a germ-free environment. In addition, all animals were cross fostered even if delivered vaginally to minimize any potential effects on microbiome composition impact on neurobehavioral outcome. However it will be important to investigate the effects of brief HI, mode of delivery, and gene risk (i.e. the "three-hit-hypothesis") on microbiome composition in a germ-free environment and determine its involvement in modulating neurobehavioural outcomes in offspring exposed to pre-term HI.

We also found significant elevations in IL-1β in the brain and blood of HI exposed offspring. IL-1β is a potent pro-inflammatory cytokine that is known to affect the activity of the hypothalamic-pituitary adrenal (HPA) axis (Maes et al., 1993). Given that the significant increases in ACTH and corticosterone in HI exposed offspring, and that systemic elevations in IL-1β in mice led to increases in plasma levels of ACTH and corticosterone (Matsuwaki et al., 2014), this suggests that activation of the HPA-axis may be through an IL-1β-dependent mechanism. Moreover, given the evidence for parallel disturbed immunity in the brain and blood in children with ASD (Estes and McAllister, 2015), and in offspring exposed to mild HI in this study, it is interesting to note that immune-related genetic networks are a much larger, intrinsic component of the wider neural-specific genetic programme that shapes the development of the nervous system of mammals (including humans) (Monzon-Sandoval et al., 2015). These findings provide a genetic basis for how there may be parallel inflammatory disturbances at the transcriptional level in both systems that are induced by a mild HI insult.

In summary, this work, adds to the growing body of evidence that perinatal complications can increase the risk of altered neurodevelopmental outcome in exposed offspring (Bohm et al. 2017, Curran et al. 2017). In future work it will be important to understand the causative molecular basis of HI-induced behavioural change. This is important to facilitate the development of targeted interventions to improve the outcome in exposed offspring in the postnatal period.

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Author Contributions

D.O'D and V.F. performed experiments. D.OD and & G.O'K. analysed data, prepared figures and wrote manuscript. All authors edited final manuscript. L.K., G.B. & G.O'K. supervised work.

Additional information

The authors declare no conflicts of interest.

Figure legends

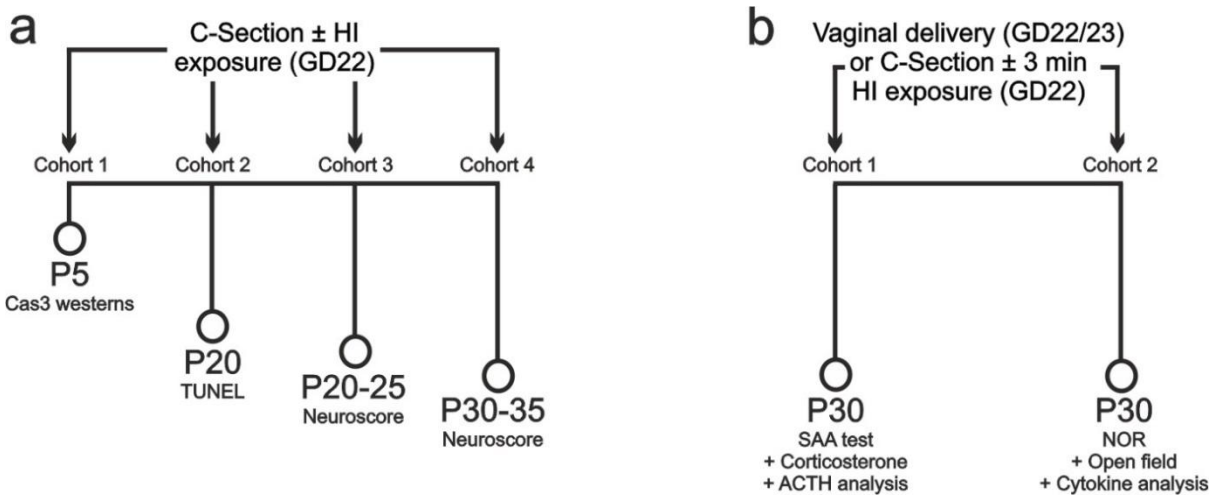


Figure 1: Schema showing the experimental design.

The infra-renal abdominal aorta and the uterine arteries of pregnant Sprague-Dawley rats (n=6 per group) were ligated on gestational day (GD) 22. The inhaled O_2 concentration was reduced to 10% for 3, 5 or 7 min. The pups were then delivered by caesarean section and cross fostered to another dam. **(a) Study 1:** Pregnant Sprague-Dawley (SD) rats were randomised (n=6 per group) to a study arm cohort which consisted of a caesarean section (c-section) with no hypoxic-ischemic (HI) injury (GD22) or c-section with 3, 5 or 7 min HI injury group (GD22). Two males from each litter was then randomized blindly into one of four cohorts: **Cohort 1:** relative expression by western blot of cleaved caspase-3 (Cas3) to pro-caspase 3 (Pro-Cas 3) in the hippocampi at postnatal day five (P5); **Cohort 2:** $Tunel^{+}$ cell count at P20; **Cohort 3:** modified 28-point neuroscore at P20-P25; and **Cohort 4:** modified 28-point neuroscore at P30-35. **(b) Study 2:** Pregnant SD rats were randomized (n=6 per group) to a study arm cohort which consisted of vaginal delivery (GD22/23), c-section with no HI injury (GD22) or c-section with 3 min HI injury (GD22). Two males from each litter was then randomized blindly into one of two cohorts: **Cohort 1:** Social approach avoidance (SAA) test and corticosterone, adrenocorticotropin hormone (ACTH) analysis at P30; and **Cohort 2:** novel object recognition (NOR) test, open field test and cytokine (IL-6, TNF-α, IL-1ß) analysis at P30.

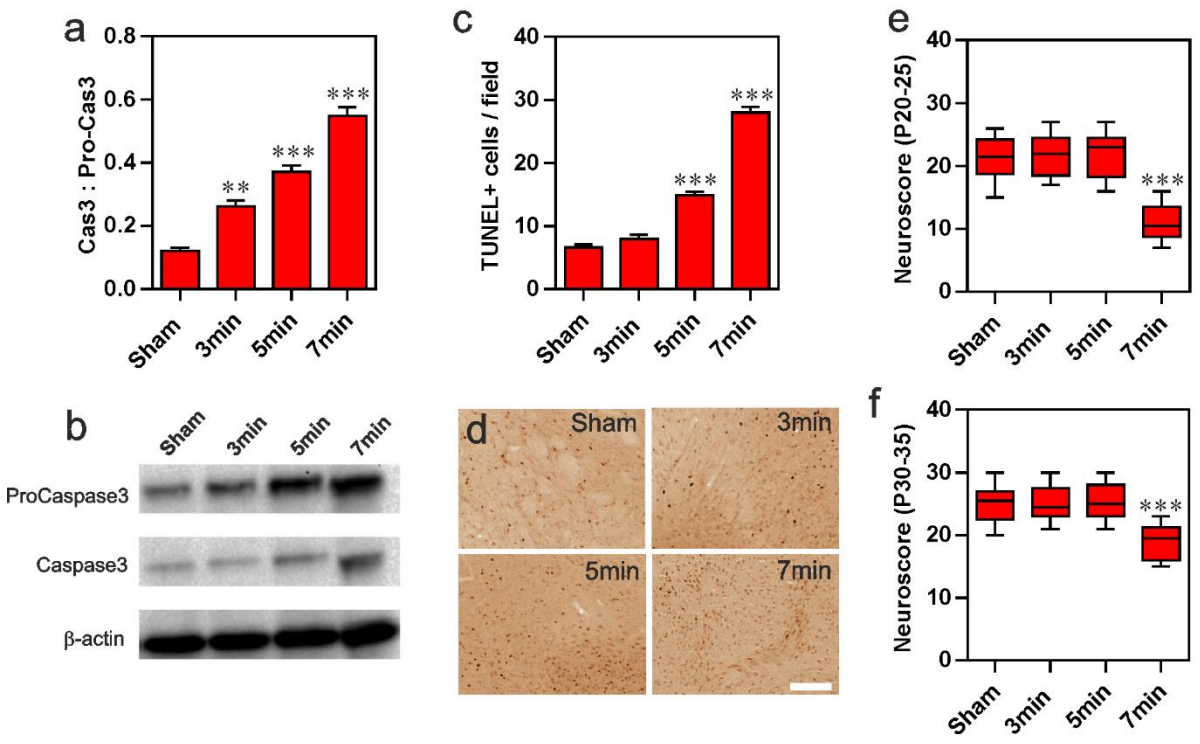


Figure 2: HI duration is the critical determinant of major neurological impairment.

(a, b) Relative expression by western blot of cleaved caspase-3 (Cas3) to pro-caspase 3 (Pro-Cas 3) in the hippocampi of affected offspring at P5. **(c)** Number of TUNEL+ cells in the brains of P20 offspring post HI exposure for 3, 5 or 7 mins. **(d)** Representative images of TUNEL immune-positive immune in the CA3 hippocampal region. ***P*<0.001, ****P*<0.0001 relative to Sham. **(e, f)** Graphs showing the results of a 28-point Neuroscore at **(e)** P20-25 and **(f)** P30-35. ****P*<0.0001 and relative to Sham. Animals used for TUNEL positive histology, western blot did not under go behaviour testing. All data are mean ± SEM. Western blot (n=3 per group, 1 pup per litter, 3 litters), TUNEL-labelled score (n=10, 2 pups per litter, 5 litters) scale bar 50 µm, neuroscore (n=10 per group, 2 pups per litter, 5 litters).

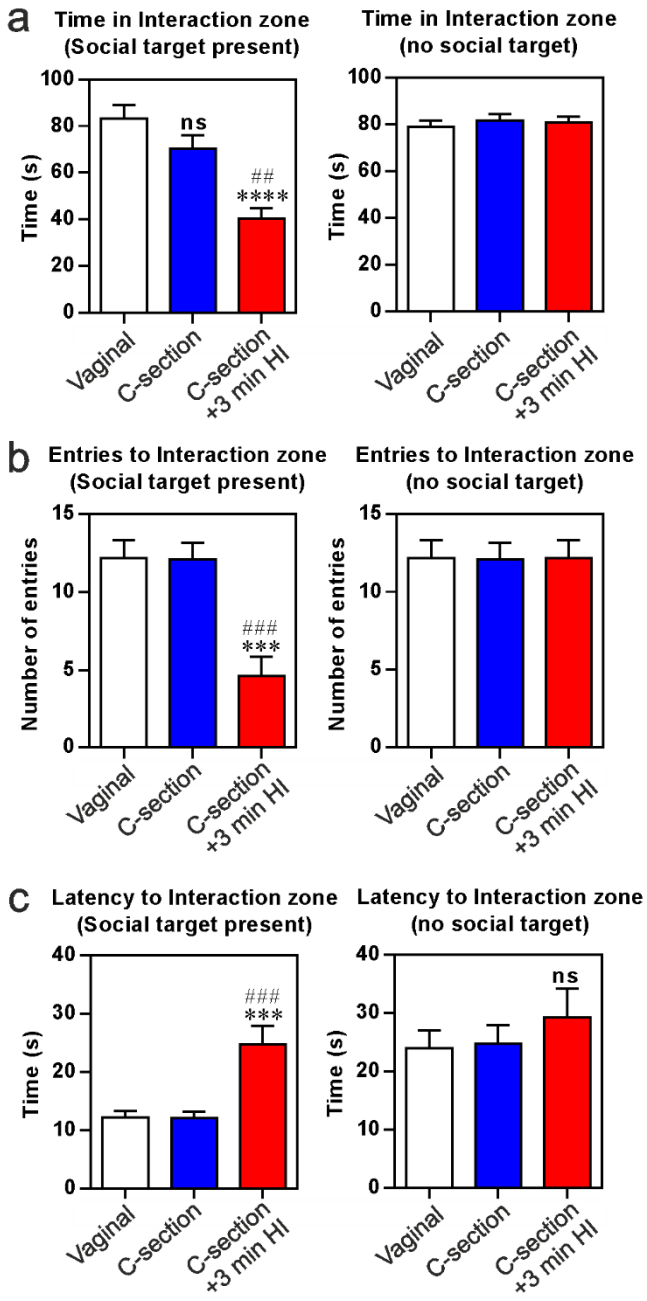


Figure 3: Mild perinatal HI exposure induces social deficits in affected offspring.

(a) Schema showing the rat social approach-avoidance (SAA) test that was carried out at postnatal day P30 in male animals (n=12) born by vaginal delivery (Cont), caesarean section (C/S) or C/S delivery post HI (C/S+HI). In the SAA test, offspring from the C/S+HI group spent, **(b)** significantly less time in the interaction zone, and (c) had a lower number of entries into the interaction zone compared to the Cont or C/S groups. There were no significant differences between any groups in **(d)** the novel objective recognition test, or **(e, f)** total distance moved in the open field test. NS = not significant, **P*<0.05 and *****P*<0.0001 relative to Cont. $^{\#}P$<0.05 and $^{\#\#}P$<0.01 comparing C/S and C/S+HI groups. One-way

analysis of variance (ANOVA) followed by a *post-hoc* Bonferroni test. All data are mean ± SEM. (10 pups (1-2 pups per litter, 5-6 litters) per group)

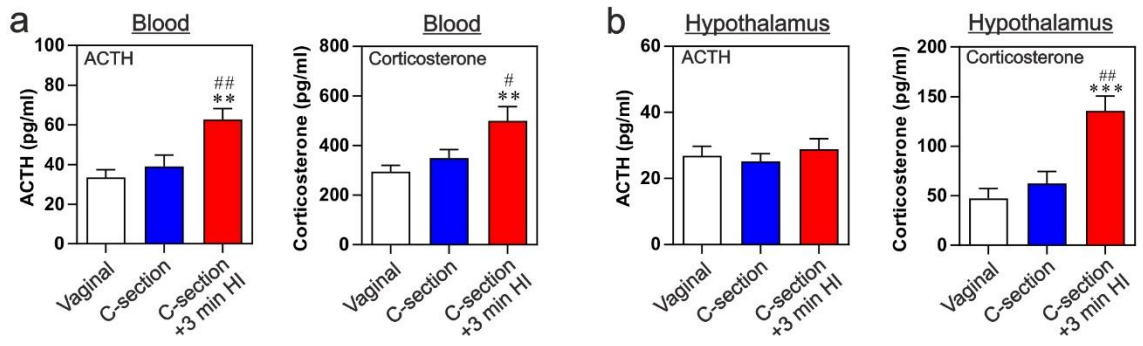


Figure 4: Perinatal HI exposure leads to an exaggerated stress response in exposed offspring.

ELISA analyses on cardiac puncture blood samples taken immediately after the SAA test for ACTH and Corticosterone in (a) a blood sample and **(b)** the hypothalamus. $^{**}P<0.01$ and $^{***}P<0.001$ relative to Cont. $^{\#}P<0.05$ and $^{\#\#}P<0.01$ comparing C/S and C/S+HI groups. All data are mean ± SEM. (8 pups (1-2 pups per litter, 5 litters) per group)

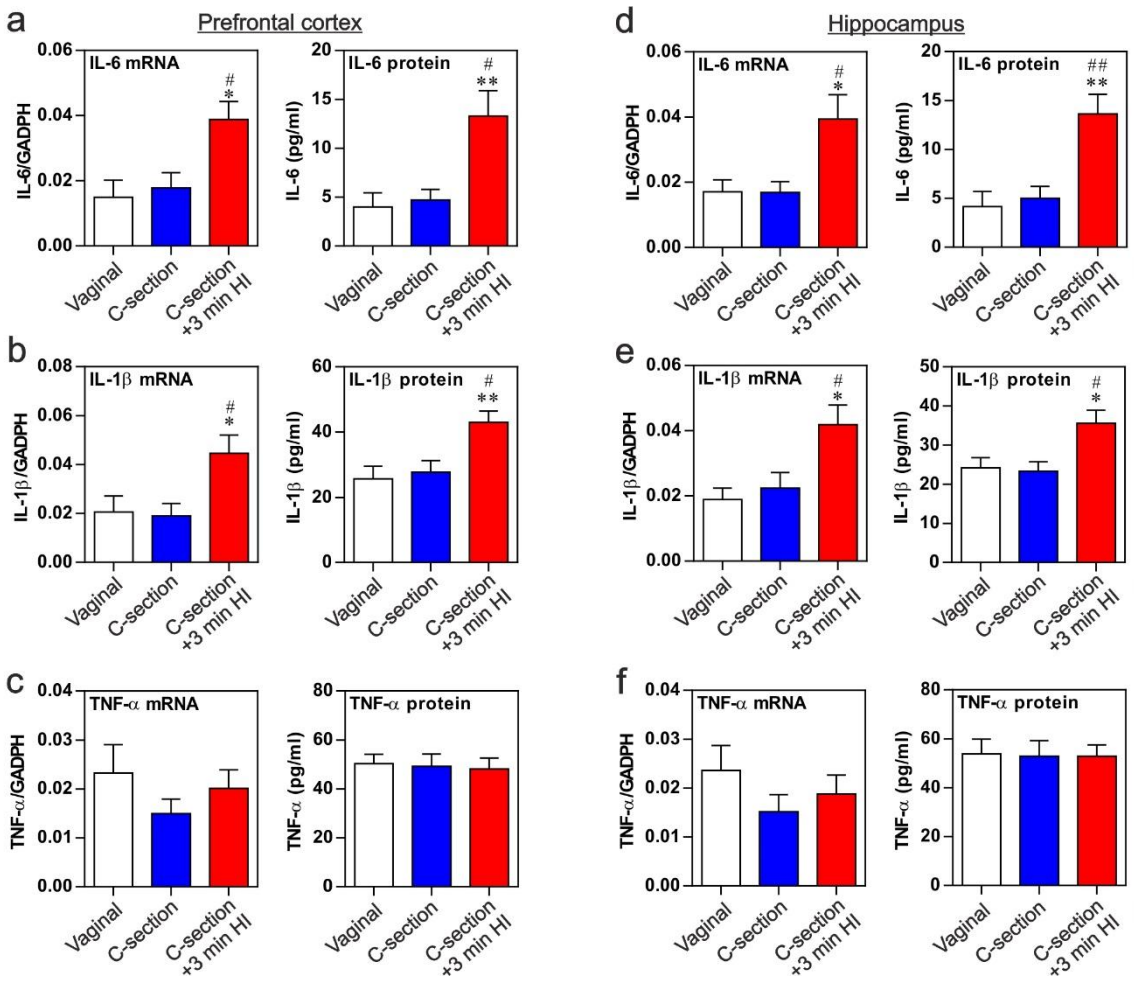


Figure 5: Elevated pro-inflammatory cytokines in the brains of exposed offspring.

Graphs showing real-time qPCR and ELISA for IL-6, IL-1ß and TNF-α mRNA and protein levels in the prefrontal cortex and hippocampus at P30. Bar charts show levels of expression of **(a, b)** IL-6, **(c, d)** IL-1β and **(e, f)** TNF-α mRNA and protein as indicated. **P*<0.05 and ***P*<0.01 relative to Cont. $^{\#}P$<0.05 and $^{\#\#}P$<0.01 comparing C/S and C/S+HI groups. All data are mean ± SEM. (8 pups (1-2 pups per litter, 5 litters) per group)

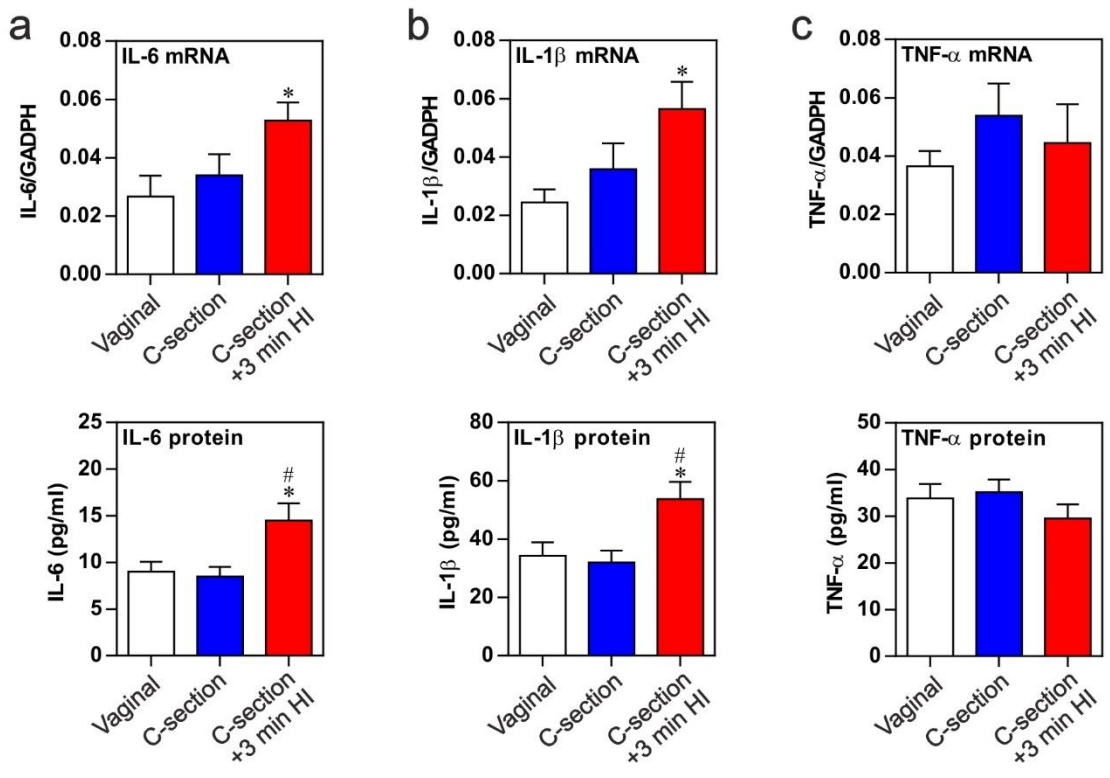
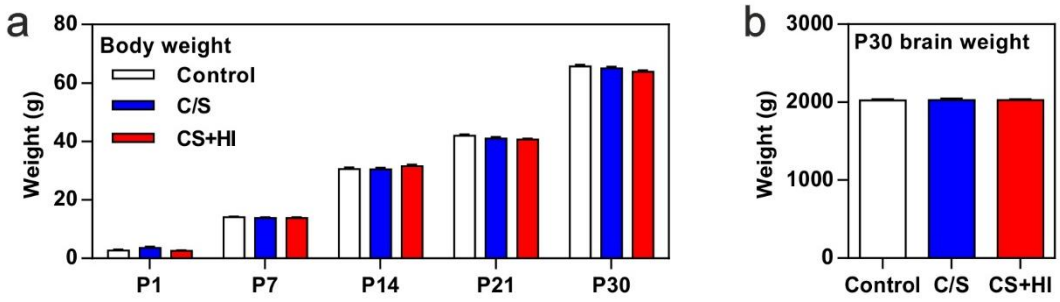


Figure 6: Elevated pro-inflammatory cytokines in the blood of exposed offspring.

Graphs showing real-time qPCR and ELISA for IL-6, IL-1ß and TNF-α mRNA and protein levels in blood samples taken at P30. Bar charts show levels of expression of **(a)** IL-6, **(b)** IL-1β and **(c)** TNF-α mRNA and protein as indicated. **P*<0.05 and ***P*<0.01 relative to Cont. $^{\#}P$<0.05 and $^{\#\#}P$<0.01 comparing C/S and C/S+HI groups. All data are mean ± SEM. (8 pups (1-2 pups per litter, 5 litters) per group)

Supplementary Figure



Supplementary Figure 1: Mild HI exposure does not affect brain or body weight.

(a) Graph showing body weight in male animals (n=10) born by vaginal delivery (Control), caesarean section (C/S) or C/S delivery post hypoxia-ischemia (HI) for 3 min (C/S+HI). No significant differences were seen in **(a)** body weight at postnatal day (P) 1, P7, P14, P21 or P30 or **(b)** brain weight at P30. One-way ANOVA followed by *post-hoc* Tukeys test. All data are presented as mean ± SEM. (10 pups (1-2 pups per litter, 5-6 litters) per group)

Tables

Table 1: Description of modified 28-point Neuroscore tests

28-point Neuroscore		
No.	**Task**	**Maximum Score**
1.	Circling	4
2.	Motility	3
3.	General Condition	3
4.	Righting reflex when positioned on back	1
5.	Paw placement of each paw on a table top	4
6.	Ability to pull self-up on a horizontal bar	3
7.	Climbing on an inclined platform	3
8.	Grip strength	2
9.	Contralateral reflex	1
10.	Contralateral rotation	2
11.	Visual forepaw reaching	2
	Maximum Score	**28**

Tables

Table 1: Description of modified 28-point Neuroscore tests

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8.	Grip strength	2
9.	Contralateral reflex	1
10.	Contralateral rotation	2
11.	Visual forepaw reaching	2
	Maximum Score	**28**